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(54) **Labelled vitamin B12 derivatives, their preparation and use.**

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Description

This invention is concerned with the assay of vitamin B_{12} and with certain labelled derivatives of vitamin B_{12} which are useful in such an assay.

The classic assay for vitamin B_{12} (hereinafter for brevity referred to simply as "B_{12}") is a microbiological assay utilising, for example, *E. gracilis*. Whilst this assay is widely accepted as accurate and reliable, it is a relatively slow method involving manual techniques. In recent years, attempts have been made to devise alternative procedures based on the well known competitive binding reaction technique, with a view to providing a quicker and equally reliable assay.

Assays based on competitive binding advantageously employ a labelled reactant, i.e. a reactant which carries an atom or group, which can readily be identified and assayed. Commonly used labels include radioactive atoms, and fluorescent or enzymic groups. There have been various proposals for making a radioactively labelled B_{12} derivative for use in a competitive binding assay of B_{12}. One proposal, which is used commercially, involves the incorporation of ^{57}Co in the biosynthesis of B_{12}. This is a complicated process and the ^{57}Co—B_{12} produced is expensive. Furthermore, the specific activity of the product is relatively low (220 μCi/μg) which, in turn, necessitates relatively lengthy counting times (of at least one minute) in competitive binding assays using this ^{57}Co—B_{12}. Low activity and long counting times are highly undesirable.

Another approach has been to prepare ^{125}I—B_{12} derivatives. U.S. patent no. 4209614 describes the preparation of such derivatives by reacting B_{12} with a glutaric anhydride derivative (which binds to the B_{12} via its sugar ring), and then iodinating the glutaric anhydride derived substituent.

A further process for preparing ^{125}I—B_{12} derivatives is described in U.S. patent no. 3981863. In this process, B_{12} is first subject to mild hydrolysis to form a mixture of monocarboxylic acids containing mostly the (e)-isomer. (The structure of vitamin B_{12} is shown in Figure 1 of the accompanying drawings.) The mixture is then reacted with a p-(aminoalkyl)phenol to introduce a phenol group into the B_{12} acids (via reaction with one of the free carboxylic acid groups). The mixed substituted B_{12} derivatives are then iodinated in the phenol-group substituent. This U.S. patent teaches that the mixed ^{125}I—B_{12} derivatives so made are useful in the radioimmunoassay of B_{12}, using antibodies raised against the mixture.

It has recently become clear (see, for example, PCT patent application US 79/00210, published as WO 79/00880) that in competitive binding assays of B_{12}, the nature of the binding protein used is of critical importance. In particular, it has been found that some binding proteins whose use has previously been suggested, not only bind to B_{12} but also to cobalamin analogues. For example, it has been shown that transcobalamin II and R protein bind to B_{12} and also to cobalamin analogues present in human sera. This lack of specificity leads to erroneous assay results. It is now generally accepted that the best binding protein to use is intrinsic factor (hereinafter "IF"), since this is highly selective for the physiologically active form of B_{12} in human sera. There is a requirement therefore for a labelled-B_{12} derivative which will bind strongly with IF.

We have investigated the mixture of ^{125}I—B_{12} derivatives described in U.S. patent no. 3981863 but have found that the mixture is not suitable for use in B_{12} assays utilising IF. The mixture is suitable (as described in the patent) where antibodies raised against the mixture are used as the binding protein, but when instead IF is used as the binding protein the assay is not accurate. We have further found that the reason for this is that the different components of the mixture surprisingly have markedly different affinities for IF. In fact we have found that, of the derivatives, those of the monocarboxylic -(d)-isomer have a very much greater affinity for IF than do the (b)- or (e)-isomer derivatives. This finding is, incidentally, in line with results reported in a paper by Kolhouse and Allen (J. Clin. Invest. *60*, 1381—1392) which is not concerned with B_{12} analysis but reports the following affinities for rabbit and human IF of the three monocarboxylic acids:

Monocarboxylic Acid Isomer	Ka B_{12}—COOH isomer / Ka Cyanocobalamin
(b)	0.0006
(d)	0.3
(e)	0.004

These affinities relate, of course, to the free acids but we have found that generally the same order of affinities is found in the derivatives thereof.

Accordingly, we have found that derivatives of the (d)-isomer, suitably radioactively labelled, are appropriate for use in assays of B_{12} in which IF is used. It is an important aspect of the present invention that labelled (d)-derivatives are used which are pure or, at least, substantially free from (b)- or (e)-isomer derivatives, since the presence of (b)- or (e)-isomer derivatives will lead to anomalous results.

The labelled derivatives of the purified B_{12} (d)-acid isomer are novel and, accordingly, in one aspect the invention provides labelled derivatives of the monocarboxylic (d)-isomer of B_{12}, in which a labelled group is

bound to the (d)-carboxylic acid group, which isomer or derivative is free from other monocarboxylic acid isomers of B_{12} and derivatives made therefrom.

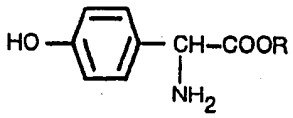
In another aspect, the invention provides a method of assaying B_{12} in a sample which comprises forming a competitive binding mixture of the sample with IF and a labelled derivative of the (d)-monocarboxylic isomer of B_{12}, in the absence of any other monocarboxylic isomer of B_{12} or derivative made therefrom. If the method of assay involves a boiling step (which is usual but is not always necessary — see our U.K. patent specification no. 2011070 the labelled (d)-isomer should be added to the reaction mixture only after the boiling step has been completed.

The labelled derivatives of the (d)-acid isomer are made by coupling to the (d)-acid group a further group which already carries, or may subsequently have attached thereto, a label. The preferred types of iodine receptors for coupling to the (d)-isomer monocarboxylic acid of B_{12} according to this invention are as follows:

1) p-aminoalkylphenols such as tyramine with the general structure

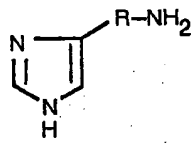
where R is an alkylene group having from 1 to 18 carbon atoms (see U.S. patent 3981863).

2) alkyl esters of tyrosine such as tyrosine methyl ester and having the general structure



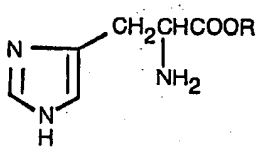
where R is an alkyl group having from 1 to 18 carbon atoms.

3) aminoalkylimidazoles such as histamine with the general structure



where R is an alkylene group having from 1 to 18 carbon atoms.

4) alkyl esters of histidine such as histidine methyl ester with the general structure



where R is an alkyl group having from 1 to 18 carbon atoms.

Derivatives of the present invention comprising iodine receptors of type 1 or 2 above are made by the method described in U.S. 3981863 (except of course that the (d)-monocarboxylic acid of B_{12} is used alone). The teachings of the said U.S. 3981863 are incorporated herein by reference. Those derivatives of the invention with iodine receptors of type 3 and 4 are made as described in the Example hereinafter.

The label may be a radioactive atom such as ^{125}I, or it may be a fluorophore or an enzyme or other label. A fluorescent-labelled isomer may be made, for example, by reacting the (d)-isomer monocarboxylic acid of B_{12}, substantially free of the (b)- and (e)-isomers, with a fluorophore, for example, fluoresceinthiocarbamyl ethylene diamine. An enzyme-labelled isomer may be made, for example, by reacting a (d)-isomer monocarboxylic acid of B_{12}, substantially free of the (c)- and (e)-isomers, with an enzyme, for example beta-galactosidase to give an enzymically labelled derivative of B_{12}. Other commonly used enzymes which could be used for this purpose include horseradish peroxidase and alkaline phosphatase.

In order to prepare the pure (d)-monocarboxylic acid isomer, B_{12} is first subjected to mild acid hydrolysis as described in U.S. 3981863 (see also J.C.S. (1953), 3848—3864). This results in the formation of a mixture of three monocarboxylic acids, namely the (b), (d) and (e) isomers. Typically, the mixture comprises 50% (e)-isomer, 25% (b)-isomer and 25% (d)-isomer. The (d)-isomer is then separated from the other isomers by the method described by Yamada *et al* (J. Biol. Chem., *247*, 6266—6270). The identity of

each of the separated isomers can be confirmed by paper chromatography using the method described by Allen *et al* (J. Clin. Invest. (1977) *60,* 1381—1392).

In order that the invention may be more fully understood, the following Example is given by way of illustration only.

Example

Preparation of monocarboxylic acids

The individual isomers were prepared and purified by the method of Yamada and Hogenkamp (J. Biol. Chem. *247,*, 6266—6270). The identity of the isomers was confirmed by paper chromatography (J. Clin. Invest. *60,* 1381—1392).

Preparation of histamine conjugates

The carboxylic acids were conjugated to histamine via an isobutylchloroformate intermediate (see Erlanger, Boreck, Beiser and Lieberman, *J. Biol. Chem.,* 228, 713 (1957)). After the reaction, the corrinoids were precipitated by addition of acetone, the precipitate re-dissolved in water and the unreacted carboxylic acid removed by passing over a 0.7 × 10 cm column of Dowex® AG1—X2 acetate. Unreacted histamine was removed by extraction into phenol and back-extraction into water (see Kolhouse and Allen, *Analyt. Biochem.* 84, 486—490 (1978)). Finally the last traces of phenol were removed by adjusting the pH to 10 with concentrated ammonium hydroxide solution and passing over a 0.7 × 10 cm of Dowex® AG1—X2 acetate. The solution was neutralised with acetic acid and stored at −20°C.

Iodination of histamine conjugates

The cobalamin-histamide conjugates were calibrated spectrophotometrically at 361 nm using a molar extinction coefficient of 27,700 and diluted to 50 µmol/L in 0.1 mol/L phosphate buffer, pH 7.5. 10 µl (0.5 nmol) of the conjugate solution was added to 20 µL (2 mCi, 1 nmol) of Na ^{125}I followed by 10 µL chloramine-T solution (1 g/L). After 5 min the reaction was stopped by the addition of 10 µl sodium metabisulphite solution (1.9 g/L). Unreacted iodide was removed by batch treatment with 50 µl Dowex® AG1—X2 acetate and the resin washed with water. The iodinated product was diluted to a concentration of 100 µg/L (based on starting concentration) in water containing 9 g/L benzyl alcohol stored at 4°C. Incorporation of ^{125}I ranged from 41% to 55% in 8 separate iodinations, giving specific activities in the range 1200—1600 Ci/g.

Binding properties of the iodinated conjugates

The iodinated materials and [^{57}Co] cyanocobalamin were tested for their ability to bind to the 10-fold excess of anti-B_{12} serum prepared as described in U.S. Patent 3981863 and to a 10-fold excess of IF under assay conditions (see below). The results are summarised in Table 1. This demonstrates that, while all three isomers are suitable for use with antibodies prepared as in U.S. Patent No. 3981863, only the (d)-isomer is suitable for use with IF.

Comparison of [^{57}Co] cyanocobalamin and [^{125}I] cyanocobalamin-d-iodohistamide in competitive protein binding assay

Intrinsic factor (<2% R-protein, from Sigma Chemicals) was dissolved in borate buffer (50 mmol/L, pH 9.5 containing 1 g/L HSA) to a concentration of 1 unit/mL. The tracers were each diluted to 50 ng/L in borate buffer containing 1 g/L dithioerythritol and 50 mg/L KCN. Each assay tube contained 0.2 mL cyanocobalamin standard, 1 mL tracer, and 0.1 mL IF. Maxmimum binding was determined by adding 0.1 ml IF at a concentration of 10 units per mL. Non-specific binding (NSB) was determined by adding buffer instead of IF. After incubation at room temperature for 1 hr, 0.5 mL albumin-coated charcoal was added. After centrifuging for 10 min at 1500 *g* the supernates were decanted into tubes and counted. The standard curves, corrected for maximum and non-specific binding (NSB) from table 1 are shown in Figure 2, in which curve A is that for [^{57}Co]-cyanocobalamin tracer and curve B is that [^{125}I] cyanocobalamin d-iodohistamide as tracer.

In the method of assay of the invention, the binding protein is preferably pure IF. However, the binding protein may be a mixture of IF and R-protein, in which mixture the R-protein has been inactivated by digestion or by B_{12} analogues which inactive the R-protein binding sites.

Table 1. Binding of labelled B_{12} (histamine conjugate) to antiserum and to purified intrinsic factor

	Using anti-B_{12} serum		Using purified intrinsic factor	
	Max binding%	NSB%	Max binding%	NSB%
$^{57}Co—B_{12}$	92.1	7.5	91.7	2.8
$^{125}I—B_{12}$-b-iodohistamide	86.5	3.8	11.3	5.2
$^{125}I—B_{12}$-d-iodohistamide	88.2	5.8	81.2	5.3
$^{125}I—B_{12}$-e-idohistamide	88.1	4.6	28.2	4.4

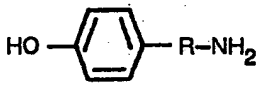
Claims

1. A derivative of vitamin B_{12} which includes an identifying label whereby the said derivative can be used in a competitive binding assay for vitamin B_{12} and quantitiated by measurement of said label, characterised in that: the derivative is prepared from the (d)-monocarboxylic acid isomer of vitamin B_{12}, and includes a labelled group bound to the (d)-carboxylic acid group, the derivative being free from other monocarboxylic acid isomers of vitamin B_{12} and derivatives thereof.

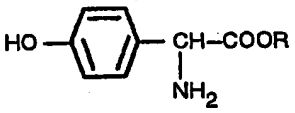
2. A derivative according to claim 1, characterised in that the labelled group contains a radioactive atom.

3. A derivative according to claim 2, characterised in that the radioactive atom is ^{125}I.

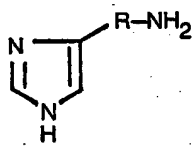
4. A derivative according to claim 3, characterised in that the labelled group is formed by binding to the (d)-carboxylic acid group one of the following compounds:
a) a p-aminoalkylphenol, such as tyramine, with the general structure:



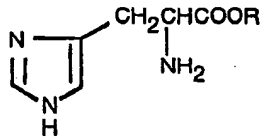
where R is an alkylene group having from 1 to 18 carbon atoms
b) an alkyl ester of tyrosine, such as tyrosine methyl ester, having the general structure:



where R is an alkyl group having from 1 to 18 carbon atoms;
c) an aminoalkylimidazole, such as histamine, with the general structure:



where R is an alkylene group having from 1 to 18 carbon atoms;
d) an alkyl ester of histidine, such as histidine methyl ester, with the general structure:



where R is an alkyl group having from 1 to 18 carbon atoms; and wherein ^{125}I is introduced into the said compound before or after binding the compound to the (d)-carboxylic acid group.

5. A derivative according to claim 1, characterised in that the labelled group comprises a fluorophore.

6. A derivative according to claim 5, characterised in that the labelled group is formed by reacting the (d)-carboxylic acid group with fluoresceinthiocarbamyl ethylenediamine.

7. A derivative according to claim 1, characterised in that the labelled group comprises an enzyme.

8. A derivative according to claim 7, characterised in that the labelled group is formed by reacting the (d)-carboxylic acid group with beta-galactidose, horseradish peroxidase or alkaline phosphatase.

9. A method of making a derivative of vitamin B_{12} as defined in claim 1, which comprises subjecting vitamin B_{12} to mild acid hydrolysis to form a mixture of monocarboxylic acid isomers thereof; and separating the (d)-isomer from the other isomers; characterised in that the separated (d)-isomer is reacted via its (d)-monocarboxylic acid group with a compound which either (1) is itself a label, or (2) which comprises a label, or (3) to which a label may subsequently be bound, and in case (3) thereafter binding a label thereto.

10. A method of assaying vitamin B_{12} in a liquid sample which comprises forming a competitive binding mixture of the sample with intrinsic factor and a labelled derivative of vitamin B_{12}, and measuring the bound or free label and therefrom determining the vitamin B_{12} in the sample, characterised in that the labelled derivative of vitamin B_{12} is as defined in any of claims 1 to 8.

11. A method according to claim 10, which includes the preliminary step of boiling the sample to denature endogenous vitamin B_{12} binding proteins therein characterised in that the said labelled derivative is mixed with the sample after the latter has been boiled.

12. A method according to claim 10 or 11, characterised in that the sample is human serum or human plasma.

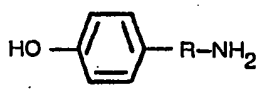
Patentansprüche

1. Markiertes Vitamin-B_{12}-Derivat, das zur Bestimmung von Vitamin B_{12} durch kompetitive Bindung und anschließende Bestimmung der Markierung verwendet werden kann, dadurch gekennzeichnet, daß es aus den (d) Monocarbonsäureisomeren des Vitamins B_{12} hergestellt ist, eine an die (d)-Carboxylgruppe gebundene Markierungsgruppe enthält sowie frei von anderen Monocarbonsäure-Isomeren von Vitamin B_{12} oder Derivaten davon ist.

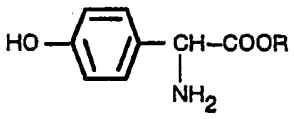
2. Derivat gemäß Anspruch 1, dadurch gekennzeichnet, daß die Markierungsgruppe ein radioaktives Atom enthält.

3. Derivat gemäß Anspruch 2, dadurch gekennzeichnet, daß das radioaktive Atom ^{125}I ist.

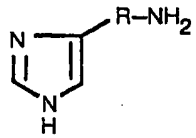
4. Derivat gemäß Anspruch 3, dadurch gekennzeichnet, daß die Markierungsgruppe dadurch hergestellt ist, daß an die (d)-Carboxylgruppe eine der folgenden Verbindungen:
(a) ein p-Aminoalkylphenol, wie Tyramin, der allgemeinen Formel:



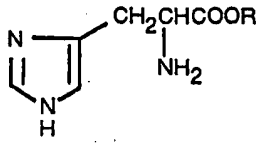
worin R eine Alkylengruppe mit 1 bis 18 Kohlenstoff-atomen ist,
b) ein Alkylester von Tyrosin, wie Tyrosinmethylester, der allgemeinen Formel:



worin R eine Alkylgruppe mit 1 bis 18 Kohlenstoff-atomen ist,
c) ein Aminoalkylimidazol, wie Histamin, der allgemeinen Formel:



worin R eine Alkylengruppe mit 1 bis 18 Kohlenstoff-atomen ist, oder
d) ein Alkylester von Histidin, wie Histidinmethylester, der allgemeinen Formel:



worin R eine Alkylgruppe mit 1 bis 18 Kohlenstoff-atomen ist, gebunden worden ist, wobei das ^{125}I in die

Verbindung vor oder nach ihrer Bindung an die (d)-Carboxylgruppe eingeführt worden ist.

5. Derivat gemäß Anspruch 1, dadurch gekennzeichnet, daß die Markierungsgruppe ein Fluorophor enthält.

6. Derivat gemäß Anspruch 5, dadurch gekennzeichnet, daß die Markierungsgruppe durch Umsetzen der (d)-Carboxylgruppe mit Fluoresceinthiocarbamyl Äthylendiamin gebildet worden ist.

7. Derivat gemäß Anspruch 1, dadurch gekennzeichnet, daß die Markierungsgruppe ein Enzym enthält.

8. Derivat gemäß Anspruch 7, dadurch gekennzeichnet, daß die Markierungsgruppe durch Umsetzen der (d)-Carboxylgruppe mit Beta-galactidose, Meerettich-Peroxydase oder alkalischer Phosphatase gebildet worden ist.

9. Verfahren zur Herstellung eines in Anspruch 1 angegebenen Derivats von Vitamin B_{12} durch Säurehydrolyse von Vitamin B_{12} unter milden Bedingungen unter Bildung eines Gemisches aus Monocarbonsäure-Isomeren davon und Abtrennen des (d)-Isomeren an den anderen Isomeren, dadurch gekennzeichnet, daß man das abgetrennte (d)-Isomere über seine (d)-Monocarbonsäuregruppe mit einer Verbindung umsetzt, die entweder (1) selbst eine Markierung darstellt oder (2) eine Markierung enthält oder (3) an die eine Markierung anschließend gebunden werden kann, wobei man im Falle von (3) anschließend eine Markierung daran bindet.

10. Verfahren zur Bestimmung von Vitamin B_{12} in einer Flüssigkeitsprobe durch Bilden eines kompetitiven Bindungsgemisches aus der Probe und Intrinsic Factor sowei einem markierten Derivat von Vitamin B_{12} bildet und die gebundene oder freie Markierung mißt und daraus den Vitamin-B_{12}-Gehalt der Probe bestimmt, dadurch gekennzeichnet, daß man als markiertes Derivat von Vitamin B_{12} eines gemäß den Ansprüchen 1 bis 8 verwendet.

11. Verfahren gemäß Anspruch 10 mit dem zusätzlichen Schritt, daß die Probe zuvor zur Denaturierung von darin enthaltenen endogenen Vitamin-B_{12}-Bindungsproteinen gekocht wird, dadurch gekennzeichnet, daß man das markierte Derivat mit de Probe vermischt, nachdem die letztere gekocht worden ist.

12. Verfahren gemäß Anspruch 10 oder 11, dadurch gekennzeichnet, daß man als Probe Humanserum oder Humanplasma verwendet.

Revendications

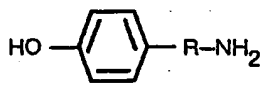
1. Un dérivé de vitamine B_{12} qui comprend un marqueur d'identification permettant d'utiliser ledit dérivé dans un dosage par fixation compétitive de la vitamine B_{12} et déterminé quantitativement par mesure dudit marqueur, caractérisé en ce que: le dérivé est préparé à partir de l'acide monocarboxylique isomère (d) de la vitamine B_{12} et comprend un groupe marqué fixé au groupe acide carboxylique (d), le dérivé étant dépourvu d'autres acides monocarboxyliques isomères de la vitamine B_{12} et de leurs dérivés.

2. Un dérivé selon la revendication 1, caractérisé en ce que le groupe marqué contient un atome radio-actif.

3. Un dérivé selon la revendication 2, caractérisé en ce que l'atome radio-actif est ^{125}I.

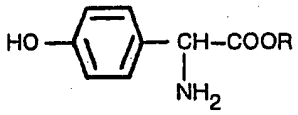
4. Un dérivé selon la revendication 3, caractérisé en ce que le groupe marqué est formé par fixation au groupe acide carboxylique (d) d'un des composés suivants:

a) un p-aminoalkylphénol, tel que la tyramine, présentant la structure générale:



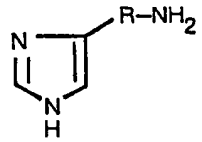
dans laquelle R est un groupe alkylène ayant 1 à 18 atomes de de carbone,

b) un ester alkylique de la tyrosine, tel que l'ester méthylique de la tyrosine, présentant la structure générale:



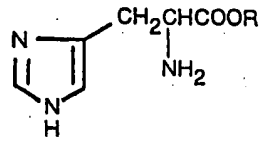
dans laquelle R est un groupe alkyle ayant 1 à 18 atomes de carbone;

c) un aminoalkylimidazole, tel que l'histamine, présentant la structure générale:



dans laquelle R est une groupe alkylène ayant 1 à 18 atomes de carbone;

d) un ester alkylique de l'histidine, tel que l'ester méthylique de l'histidine, présentant la structure générale:



dans laquelle R est un groupe alkyle ayant 1 à 18 atomes de carbone;
et le ^{125}I est introduit dans ledit composé avant ou après la fixation du composé au groupe acide carboxylique (d).

5. Un dérivé selon la revendication 1, caractérisé en ce que le groupe marqué comprend un fluorophore.

6. Un dérivé selon la revendication 5, caractérisé en ce que le groupe marqué est formé par réaction du groupe acide carboxylique (d) avec la fluorescéinethiocarbamyléthylènediamine.

7. Un dérivé selon la revendication 1, caractérisé en ce que le groupe marqué comprend une enzyme.

8. Un dérivé selon la revendication 7, caractérisé en ce que le groupe marqué est formé par réaction du groupe acide carboxylique (d) avec la β-galactosidase, la peroxydase de raifort ou une phosphatase alcaline.

9. Un procédé de préparation d'un dérivé de vitamine B_{12} comme défini dans la revendication 1 qui consiste à soumettre la vitamine B_{12} à une hydrolyse acide ménagée pour former un mélange des acides monocarboxyliques isomères; et à séparer l'isomère (d) des autres isomères; caractérisé en ce que l'isomère (d) séparé est mis à réagir par l'intermédiaire de son groupe acide monocarboxylique (d) avec un composé qui soit (1) est lui-même un marqueur, soit (2) comprend un marqueur, soit (3) auquel un marqueur peut ultérieurement être fixé et dans le cas (3) la fixation ultérieure d'un marqueur à celui-ci.

10. Un procédé de dosage de la vitamine B_{12} dans un échantillon liquide qui comprend la formation d'un mélange de fixation compétitive de l'échantillon avec le facteur intrinsèque et un dérivé marqué de la vitamine B_{12} et la mesure du marqueur fixé ou libre et la détermination à partir de cette mesure de la vitamine B_{12} dans l'échantillon, caractérisé en ce que le dérivé marqué de la vitamine B_{12} est comme défini dans l'une quelconque des revendications 1 à 8.

11. Un procédé selon la revendication 10 qui comprend le stade préliminaire d'ébullition de l'échantillon pour y dénaturer les protéines endogènes fixant la vitamine B_{12}, caractérisé en ce que ledit dérivé marqué est mélangé à l'échantillon après que ce dernier ait été porté à ébullition.

12. Un procédé selon la revendication 10 ou 11, caractérisé en ce que l'échantillon est un sérum humain ou un plasma humain.

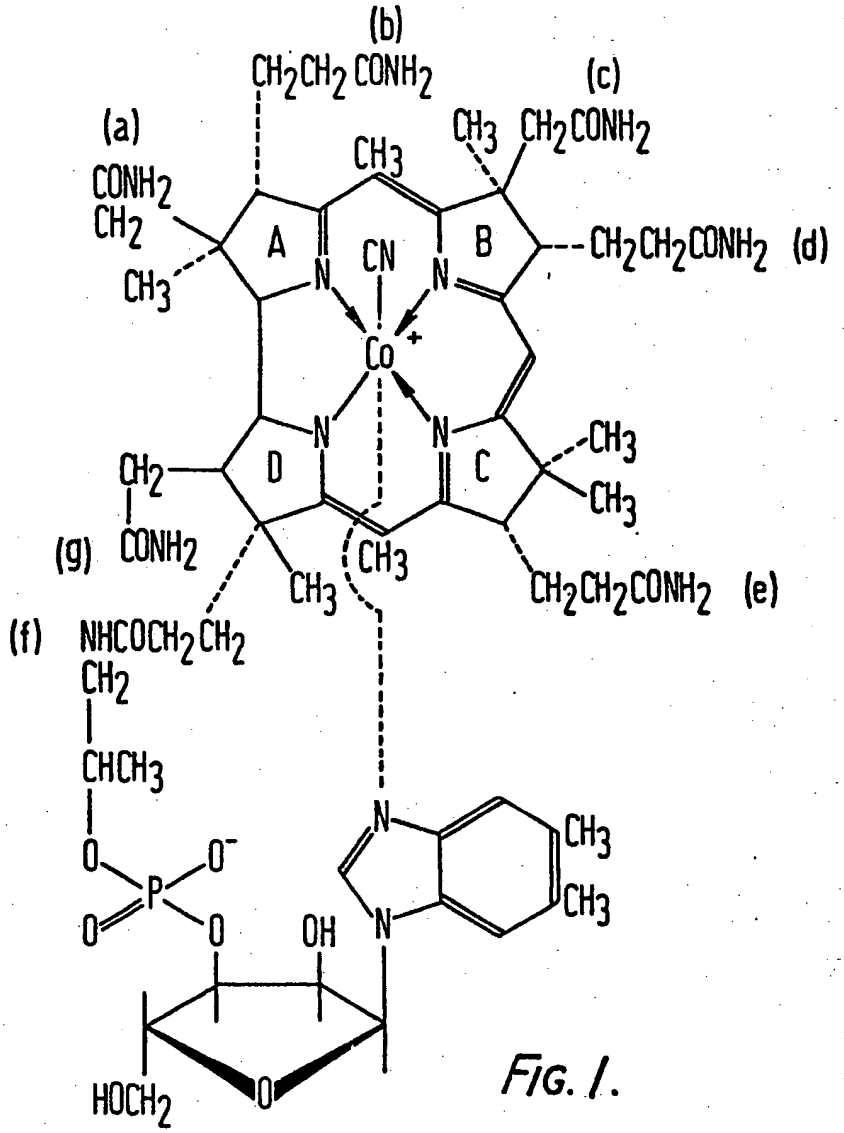

(b)
CH2CH2 CONH2
(c)
(a)
CH3
CH3 CH2CONH2
CONH2
CH2
A
N
CN
N
B
CH2CH2CONH2 (d)
CH3
Co+
N
N
CH3
CH2
D
C
CH3
(g) CONH2
CH3
CH3
CH2CH2CONH2 (e)
(f) NHCOCH2CH2
CH2
CHCH3
O
O⁻
P
O
O
OH
N
CH3
N
CH3
HOCH2
O

FIG. 1.

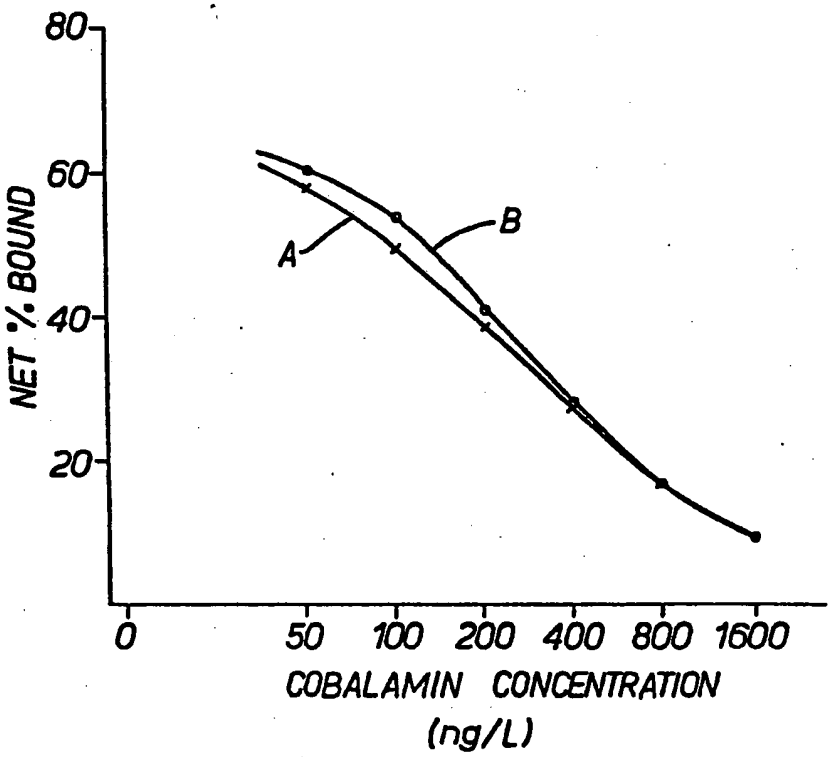

80
60
40
20
NET % BOUND
A
B
0
50
100
200
400
800
1600
COBALAMIN CONCENTRATION
(ng/L)

FIG. 2.

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